



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 5 :</b> <b>A61K 37/02, C07K 5/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 92/13549</b> <b>(43) International Publication Date:</b> 20 August 1992 (20.08.92)
<b>(21) International Application Number:</b> PCT/US92/00905 <b>(22) International Filing Date:</b> 4 February 1992 (04.02.92) <b>(30) Priority data:</b> 652,161 7 February 1991 (07.02.91) US <b>(71) Applicant:</b> RESEARCH CORPORATION TECHNOLOGIES, INC. [US/US]; 6840 East Broadway Boulevard, Tucson, AZ 85710 (US). <b>(72) Inventors:</b> HATHAWAY, David, R. ; 7966 N. Illinois Street, Indianapolis, IN 46260 (US). MARCH, Keith, L. ; 113611 Springmill Blvd., Carmel, IN 46032 (US).	<b>(74) Agent:</b> SCOTT, Anthony, C.; Scully, Scott, Murphy and Presser, 400 Garden City Plaza, Garden City, NY 11530 (US). <b>(81) Designated States:</b> AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent). <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> INHIBITION OF CELL PROLIFERATION BY HYDROPHOBIC PEPTIDES  <b>(57) Abstract</b> <p>The present invention is directed a method of using certain hydrophobic peptides for the inhibition of cell proliferation, wherein the peptides have the general formula: R-Xaa<sub>1</sub>-(Xaa)<sub>m</sub>-Xaa<sub>n</sub>. The subject peptides have 2-7 amino acids such as alanine (Ala), arginine (Arg), cysteine (Cys), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), norleucine (nLeu), phenylalanine (Phe), proline (Pro), threonine (Thr), tyrosine (Tyr), tryptophan (Trp), or valine (Val). In accordance with the present invention, these peptides are potent inhibitors of cell proliferation as well as inhibitors of the synthesis of two cellular proto-oncogenes. One aspect of the present invention provides for the prevention and treatment of cancer by administration of the subject peptides. A further aspect of the present invention provides for inhibiting cell proliferation using the subject peptides in the treatment and prevention of prostatic hypertrophy, arterial occlusion (restenosis), arteriosclerosis, and smooth muscle cell diseases.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark	MG	Madagascar		
ES	Spain				

INHIBITION OF CELL PROLIFERATION  
BY HYDROPHOBIC PEPTIDES

1

Inappropriate, inopportune or excessive cell proliferation is a major cause not only of cancer but also of heart disease. Typically a cancerous cell loses its ability to respond to cellular signals for growth restraint. Similarly, unchecked smooth muscle cell growth within the inner lining of arteries is a major cause of arteriosclerosis and of reocclusion after arterial dilation.

10

The present invention is directed towards the use of certain hydrophobic peptides to inhibit cellular growth, especially of smooth muscle cells and cancerous cells. Generally these hydrophobic peptides have 2-7 amino acids such as alanine (Ala), arginine (Arg), cysteine (Cys), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), norleucine (nLeu), phenylalanine (Phe), proline (Pro), threonine (Thr), tyrosine (Tyr), tryptophan (Trp) or valine (Val). In accordance with the present invention, these peptides are potent inhibitors of cell proliferation.

15

20

One aspect of the present invention provides for the prevention and treatment of cancer by administering the subject peptides to an animal.

25

Another aspect of the present invention relates to inhibiting cell proliferation of smooth muscle cells with the subject peptides. An additional aspect of this invention relates to the prevention of arterial occlusion in vivo using the subject peptides and methods of administration of these peptides for the prevention and treatment of arteriosclerosis.

30

35

-2-

1           Biologists have long recognized that control of  
cell proliferation is one of the most basic aspects of  
multicellular existence. Throughout embryological  
development, and through all of adult life,  
5   differentiated cells have a choice of whether to divide  
or not. Only if a programmed series of correct decisions  
is made can the organism continue to function normally.  
If incorrect decisions are made one cell type can  
replicate unchecked, thereby interfering with critical  
10   life functions and ultimately threatening the existence  
of the organism.

          Intertwined with any discussion or study of  
cell proliferation is the nature and basis of cancer.  
This collection of horrific diseases by definition  
15   involves cells which divide when they should not, thus  
producing tumors. Cancer can arise by changes in  
differentiated cell types, resulting in cancer cells that  
exhibit many of the morphological and functional  
characteristics of their respective non-cancerous  
20   precursor cells. Cancer is common in cells that normally  
undergo frequent division (e.g. epithelial cells of the  
skin) and so the problem may not be that cancer cells  
divide frequently but that they lack the normal control  
systems to stop unwanted cell division. Hence, in an  
25   effort to find effective drugs for cancer therapy,  
reagents are sought which control or inhibit cell  
proliferation. The peptides of the present invention can  
provide this control.

          However, cancer is not the only disease that is  
30   exacerbated by excessive cell proliferation. Researchers  
also recognize that unchecked proliferation of smooth

35

1 muscle cells can lead to partial blockage of arteries,  
causing arteriosclerosis. Even after treatment of  
arteriosclerosis by balloon dilation (angioplasty) of  
blocked portions of an arteries, further smooth muscle  
5 cell proliferation can cause reocclusion (restenosis) of  
the artery. Hence, inhibition of smooth muscle cell  
proliferation in arteriosclerosis, or after dilation of  
arteries, provides a promising new treatment strategy.

Arteriosclerosis is a disease of the inner  
10 lining, or intima, of arteries leading to formation of  
fatty lesions on the arterial inner surfaces. The  
earliest stage in the development of these lesions is  
believed to be damage to the endothelial cells and  
sublying intima. Damage can be caused by physical  
15 abrasion of the endothelium, by abnormal substances in  
the blood, or even by the effect of the pulsating  
arterial pressure on the vessel wall. Once the damage  
has occurred, smooth muscle cells proliferate and migrate  
from the media (middle layer) of the arteries into the  
20 lesions. Soon thereafter, lipidic substances, especially  
cholesterol, begin to deposit within the proliferating  
muscle cells, generating plaques. In later stages,  
synthesis of extracellular matrix by fibroblasts and  
other cells infiltrating the degenerative areas causes  
25 progressive sclerosis (fibrosis) of the arteries.  
Calcium often precipitates with lipids to generate  
calcified plaques. When fibrosis and calcification  
occurs, the arteries become extremely hard. The hardened  
arteries lose most of their distensibility, and the  
30 region within and surrounding them is easily ruptured.  
Arteriosclerotic plaques often protrude through the

-4-

1 intima into the flowing blood, and the biochemical or  
physical changes of the plaque surface can cause blood  
clots to develop.

5 Almost half of all human beings die of arterio-  
sclerosis. Approximately two thirds of these deaths are  
caused by clot formation in a coronary artery, the  
remaining one third occur by clot formation, or  
hemorrhage of vessels, in other organs of the body,  
especially in the brain (causing strokes).

10 A commonly used treatment for coronary arterio-  
sclerosis is percutaneous transluminal coronary  
angioplasty, or arterial dilation. This procedure is a  
treatment of choice because it can enlarge a narrowed  
arterial passageway. However, arterial injury may occur  
15 during angioplasty and this injury can re-initiate or  
intensify the process of plaque formation leading to  
reocclusion of the artery. In fact reocclusion  
(restenosis) of the artery appears to be an exaggerated  
response to the controlled injury of angioplasty, and  
20 occurs in 30-40% of all patients receiving angioplasty.  
The process of restenosis is very similar to the  
formation of the original arteriosclerotic plaque, but  
occurs on a shorter time scale. Endothelial cell injury  
caused by angioplasty leads to platelet aggregation and  
25 shortly thereafter to activation of smooth-muscle cell  
proliferation. Platelets secrete platelet-derived growth  
factor (PDGF), which is one of the most potent cell  
proliferative factors for smooth muscle cells found in  
serum. PDGF is also a chemotactic attractant for smooth  
30 muscle cells and may be responsible for attracting  
smooth muscle cells from the middle layer, or media, of

35

1 the artery into the intima as well as for the initial  
proliferation of smooth muscle cells within the intima.  
However, it is thought that PDGF alone does not optimally  
stimulate DNA synthesis. PDGF-stimulated cells require a  
5 second group of growth factors (termed "progression  
factors"), to initiate DNA synthesis and cell division.  
PDGF alone appears to simply stimulate cells to enter a  
new cell cycle by causing the cell to move from the  $G_0$   
arrest state to  $G_1$  (Fig. 1 depicts the time course of a  
10 typical mammalian cell cycle). Exposure to progression  
factors allows cells to move through the cell cycle by  
initiating DNA synthesis (S phase). A number of  
progression factors are known, including epidermal growth  
factors from platelets and somatomedin-C present in  
15 serum.

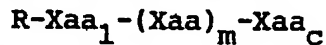
In accordance with the present invention,  
specific peptides are shown to be effective in the  
inhibition of cell proliferation, and provide new  
reagents for the prevention and treatment of  
20 arteriosclerosis, prostatic hypertrophy and various forms  
of cancer. The block in cell division caused by the  
present peptides appears to occur prior to the S, or DNA  
synthesis, phase of the cell cycle. A secondary block to  
cell division in the  $G_2$  or M phase of the cell cycle, is  
25 also observed when cells are exposed to the subject  
peptides for longer periods of time.

The subject peptides are also inhibitors of  
calcium-dependent thiol proteases, calpains I and II.  
The calpains are cytosolic cysteine proteases which are  
30 ubiquitously distributed in most cell types. The  
biological function of calpains is not clear. However,

-6-

- 1 calpains have been implicated in numerous processes  
including the re-structuring of the plasma membrane  
(Zaidi, et al., 1989, J. Membrane Biol. 110:209-216), the  
5 proteolysis of two important cytoskeletal proteins  
(Yoshida, et al., 1984, FEBS Letters 170:259-262) as well  
as the regulation of platelet aggregation (Fox, et al.,  
1983, J. Biol. Chem. 258:9973-9981) and the activation of  
several cellular regulatory proteins (Murachi et al.,  
1981, in Advances in Enzyme Regulation, G. Weber, ed.,  
10 Pergamon Press, New York, 19:407-424).

The present invention is directed to a method  
of inhibiting animal cell proliferation, especially the  
inappropriate, inopportune, or excessive cell  
proliferation associated with cancer, arteriosclerosis,  
15 restenosis, and smooth muscle or endothelial cells. In  
particular, the method provides for administering a  
growth inhibiting amount of at least one of certain  
hydrophobic peptides to an animal or to cultured cells,  
wherein the subject peptides range from 2 to 7 amino acids  
20 and are represented by the formula:



and further wherein

m is 0-5;

R is hydrogen, epoxysuccinyl, cholesteryl,  
25 aryl, aralkyl or acyl;

Xaa<sub>1</sub> and (Xaa)<sub>m</sub> are independently Ala, Arg,  
Ile, Leu, Lys, Met, nLeu, Phe, Pro, Thr, Tyr, Trp or Val;

Xaa<sub>C</sub> is an amino acid from the group Ala, Arg,  
Cys, Ile, Leu, Lys, nLeu, Phe, Pro, Thr, Tyr, Trp, Val or  
30 the corresponding alcohol, aldehyde, epoxysuccinate, acid

-7-

1 halide, carbonyl halomethane or diazomethane derivative  
of the carboxy terminal group of said amino acid.

Specifically, the preferred peptides of this  
invention include shorter peptides with two or three  
5 amino acids, i.e. m being 0 or 1, and more especially  
include benzyloxycarbonyl-Leu-norleucinal and  
acetyl-Leu-Leu-  
norleucinal.

Moreover, the present method is useful in  
10 treating and preventing smooth muscle cell proliferation,  
arteriosclerosis, restenosis, especially restenosis  
occurring after percutaneous transluminal coronary  
angioplasty, and cancer and cancerous-like conditions  
such as prostatic hypertrophy, small cell carcinoma of  
15 the lung and some endotheliomas and sarcomas.

Another aspect of the invention provides  
pharmaceutical compositions containing the subject  
peptides with a pharmaceutically acceptable carrier for  
administration to an animal in accordance with the  
20 methods of this invention.

Fig. 1 depicts the phases of the mammalian cell  
cycle and the approximate duration of each phase.

Fig. 2 depicts the inhibitory effect of  
calpeptin (benzyloxycarbonyl-leucine-norleucinal) on the  
25 proliferation of vascular smooth muscle cells. The open  
circles depict the growth of untreated cells; the solid  
circles depict the growth of cells treated with  
calpeptin.

Fig. 3 depicts the normal distribution of DNA  
30 in cultured vascular smooth muscle cells at various times

35

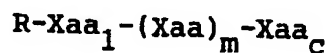
1 after addition of serum to serum depleted cells. At 12  
hours (T=12 hr) after serum addition most cells have a 2n  
DNA content (the amount of DNA normally found in resting  
or non-dividing cells). Addition of serum allows the  
5 cells to enter the cell cycle and to begin DNA synthesis.  
By 24 hours (T=24 hr) after serum addition there is an  
approximate equal distribution of cells with a 2n DNA  
content and a 4n DNA content (twice the amount of DNA as  
in non-dividing cells).

10 Fig. 4 depicts a comparison of the amount of  
DNA in cells exposed to calpeptin with that in cells not  
exposed to calpeptin. This figure demonstrates that  
exposure to calpeptin arrests the division of most cells  
prior to DNA synthesis, since most cells exposed to  
15 calpeptin have a 2n content of DNA after 24 hours of  
culture in the presence of serum. The top left panel  
provides a control of dividing cells for comparison with  
the other panels: a 2n DNA content is depicted by the  
left peak and a 4n DNA content is depicted by the right  
20 peak. Chronic exposure of cells to calpeptin allows some  
progression through the cell cycle to the G<sub>2</sub> or M phase  
of the cell cycle as demonstrated by a higher DNA content  
in cells after 2 weeks exposure to calpeptin.

Fig. 5 depicts the effect that acetyl-Leu-Leu-  
25 norleucinal (Inhibitor I), acetyl-Leu-Leu-methioninal  
(Inhibitor II) and calpeptin have on DNA synthesis as  
measured by <sup>3</sup>H-thymidine incorporation in growing smooth  
muscle cells. The amount of thymidine incorporated into  
cells treated with 10<sup>-5</sup> to 10<sup>-4</sup>M of calpeptin or  
30 acetyl-Leu-Leu-norleucinal is significantly less than  
that incorporated into control cells.

-9-

1           The present invention provides a method of  
 inhibiting cell proliferation in vivo or in vitro, using  
 a class of hydrophobic peptides which are effective for  
 that purpose. A general formula depicting the structures  
 5 of these peptides is:



wherein:

10           m is 0-5;  
             R is hydrogen, an epoxysuccinyl, a cholesteryl,  
             aryl, aralkyl or acyl;

            Xaa<sub>1</sub> and Xaa<sub>m</sub> are independently Ala, Arg, Ile,  
 15           Leu, Lys, norleucine (nLeu), Phe, Pro, Thr, Tyr, Trp, or  
             Val;

            Xaa<sub>c</sub> is an amino acid from the group Ala, Arg,  
             Cys, Ile, Leu, Lys, nLeu, Phe, Pro, Thr, Tyr, Trp, Val or  
             the corresponding aldehyde, alcohol, epoxysuccinate, acid  
 20           halide, carbonyl halomethane, or diazomethane derivative  
             of the carboxy terminal amino acid.

            Xaa<sub>1</sub> is the N-terminal amino acid; Xaa<sub>c</sub> is the  
             C-terminal amino acid and (Xaa)<sub>m</sub> represents internal  
             amino acids, if present. The values of m range from 0 to  
             5, with preferred values of m being 0 to 3. The most  
 25           preferred values for m are 0 or 1.

            The aldehyde, alcohol, epoxysuccinate, acid  
             halide, carbonyl halomethane, or diazomethane  
             carboxy-terminal derivatives of Xaa<sub>c</sub> are represented by  
             the formulas  $-\text{CH}=\text{O}$ ,  $-\text{CH}_2-\text{OH}$ ,  $-\text{CO}-\text{CH}(\text{O})-\text{CH}-\text{CO}_2^-$ ,  $-\text{CO}-\text{Y}$ ,  
 30

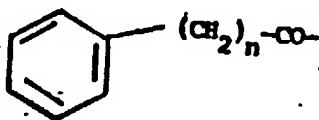
35

1     $-\text{CO}-\text{CH}_2-\text{Y}$ , or  $-\text{CH}-\text{N}_2$  respectively. As used herein, Y is a  
halo group, or halide, especially Cl, Br or I. In  
particular, Cl or Br are preferred with Cl as the most  
preferred.

5           When R is an acyl group it has the general  
formula  $\text{R}'-\text{CO}$ , wherein R' is a lower alkyl or aryl group.

          As used herein, the term lower alkyl refers to  
alkyl groups containing one to six carbon atoms. These  
groups may be straight-chained or branched and include  
10 such moieties as methyl, ethyl, propyl, isopropyl,  
n-butyl, sec-butyl, isobutyl, t-butyl, pentyl, amyl,  
hexyl and the like. The preferred alkyl groups are  $\text{C}_1-\text{C}_4$   
alkyl.

          When R or R' is an aryl group then the term  
15 aryl, when used alone or in combination refers to an  
aromatic ring containing six to ten carbon atoms.  
Moreover, the present aryl groups include aralkyl groups  
(aryl groups with lower alkyl groups as ring  
substituents) and more specifically the groups benzyl,  
20 benzoyl, naphthyl, carboxybenzyl, benzyloxycarbonyl, or



25           wherein n is an integer from 0-6 and preferably 0-3.  
Aryl groups may have lower alkyl groups substituents at  
any, some or all, available ring positions. Lower alkyl  
group substituents are the same as those defined above.

30           Preferred R groups are cholesteryl,  
benzyloxycarbonyl, acetyl or benzoyl groups. The most

35

-11-

1 preferred R groups are acetyl, benzyloxycarbonyl and  
cholesteryl.

For Xaa<sub>1</sub> the preferred amino acids are Arg,  
Ile, Leu, Lys, Met, nLeu, Phe, Tyr or Val. The most  
5 preferred are Leu nLeu or Val.

Each (Xaa)<sub>m</sub> amino acid can independently be  
Ala, Arg, Ile, Leu, Lys, Met, nLeu, Phe, Pro, Tyr, Trp or  
Val. Preferred Xaa<sub>m</sub> amino acids are Arg, Ile, Leu, Lys,  
Met, nLeu, Phe, Thr, Trp, Tyr, or Val. The most  
10 preferred (Xaa)<sub>m</sub> amino acids are Leu, nLeu, or Val.

Preferred Xaa<sub>c</sub> amino acids are the aldehyde,  
alcohol, epoxysuccinate, acid halide, carbonyl  
halomethane or diazomethane derivatives of Arg, Ile, Leu,  
Lys, nLeu, Phe, Thr, Trp, Tyr or Val. The most preferred  
15 carboxy-terminal amino acids are the aldehyde derivatives  
of Leu, Lys, nLeu, Phe or Tyr.

In particular, the preferred hydrophobic  
peptides of this invention are calpeptin  
(benzyloxycarbonyl-Leu-norleucinal) and acetyl-Leu-Leu  
20 norleucinal.

The subject peptides may be chemically  
synthesized or isolated from a bacterial, fungal or plant  
source. Isolation can be by any technique used by one  
skilled in the art, including differential extraction,  
25 ion-exchange or gel filtration column chromatographic  
procedures and high pressure liquid chromatography.  
Chemical synthesis of the subject peptides is by any of  
the methods for peptide synthesis, for example by either  
solution or solid phase synthetic procedures such as the  
30 Merrifield procedure. Solid phase synthesis is commonly  
preferred for making longer peptides but many of the

35

- 1 short peptides (with two to four amino acids) can be made  
efficiently by solution synthesis.

A basic problem in peptide synthesis is one of  
blocking or protecting the alpha amino group from  
5 indiscriminate reaction with a carboxyl group of an  
undesired amino acid; an additional problem is the  
prevention of reactions with amino acid side chains.  
These undesirable side reactions are prevented by use of  
blocking groups that render an alpha amino group, or a  
10 side chain group, unreactive but permit the desired  
reaction to take place. In addition to providing  
protection against undesirable reactions, the blocking  
group must be easily removed without chemically altering  
the remainder of the molecule, especially the peptide  
15 linkage that has been built up during synthesis. (See  
generally, Morrison and Boyd, Organic Chemistry, Third  
Ed., Sec. 30.10 Synthesis of Peptides, pp. 1131-1133,  
1983).

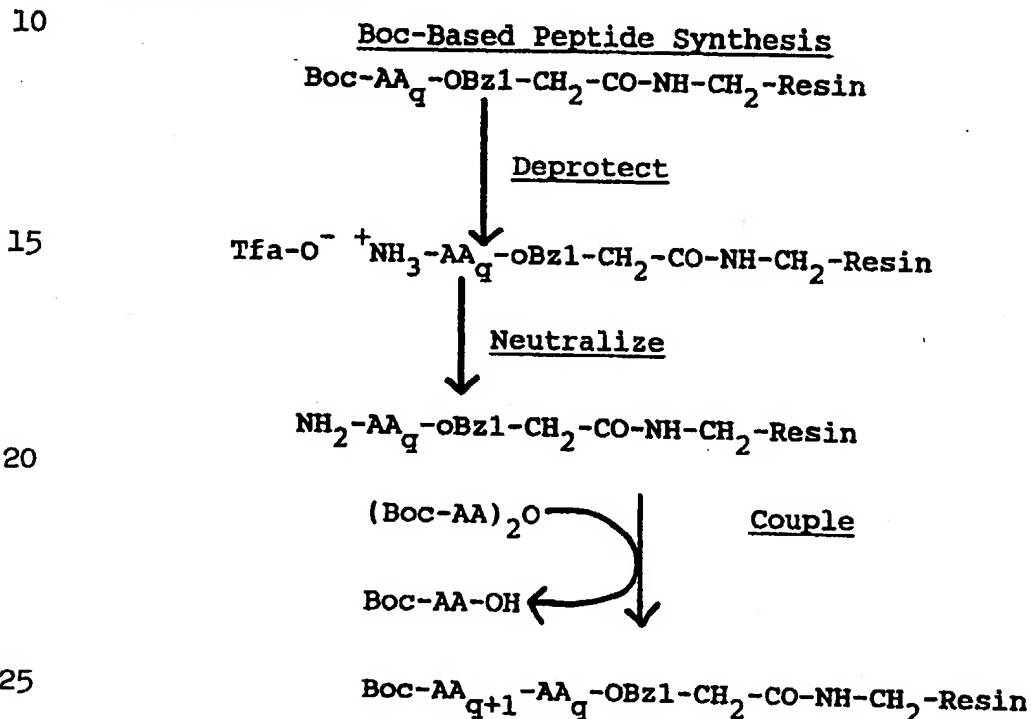
In practice, the strategy of maximal protection  
20 of all side chain functionalities has been routinely  
employed. There are at least two sets of side  
chain-protecting groups and the choice of one set over  
another is dictated by the protecting group strategy  
chosen for the alpha amino group. General procedures for  
25 peptide synthesis are provided in Barany et al. (1980, in  
The Peptides 2: 1-284, Gross E. and Meienhofer, J. eds,  
Academic Press, New York) and Stewart et al. (Solid Phase  
Peptide Synthesis, Pierce Chemical Co.).

Commonly used alpha amino protecting groups are  
30 tert-butyloxycarbonyl (Boc; cleaved by acid treatment),  
9-fluorenylmethyloxycarbonyl (Fmoc; removed by treatment

-13-

1 with a secondary amine such as piperidine) and carbobenzyoxy (Z or Cbz; removed by catalytic hydrogenation).

5 The alpha-amino Boc, benzyl-based side chain protection strategy relies on the principle of graduated acid lability of the protecting groups. Boc-protected amino acids are inexpensive and available in high purity. A general scheme for Boc-based peptide synthesis is depicted below.



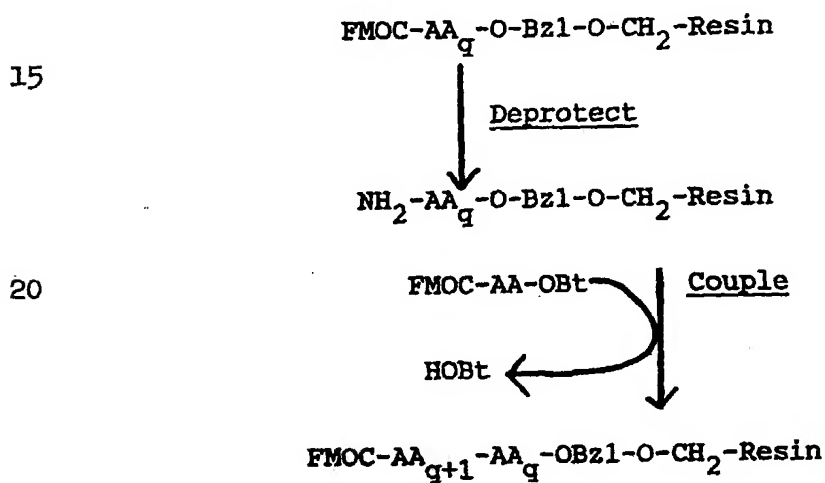
30 In the Boc-based peptide synthetic scheme depicted above, Boc-AA represents an amino acid with a Boc protecting group on its alpha amino group. The subscript q on AA depicts the number of amino acids in the peptide; q is an integer, from 1-6 in the present

35

- 1 invention. Tfa-O<sup>-</sup> is trifluoroacetic acid.  
 OBzl-CH<sub>2</sub>-CO-NH-CH<sub>2</sub>-Resin represents a possible coupling  
 arm and its attachment to a solid phase resin. As  
 described above, use of a solid phase resin is optional,  
 5 depending on the size of the peptide.

The FMOC based strategy uses different  
 mechanisms for removal of the alpha amino and side chain  
 protecting groups: a secondary amine for the alpha amino  
 protecting group and treatment with trifluoroacetic acid  
 10 for the side chain protecting groups. An FMOC-based  
 peptide synthesis scheme is depicted below.

FMOC-Based Peptide Synthesis

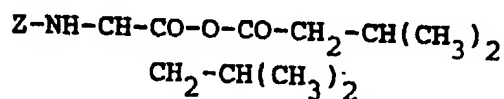
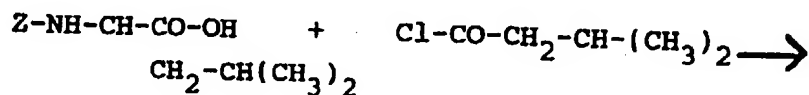


An FMOC protected amino acid is represented by FMOC-AA.  
 The O-Bzl-O-CH<sub>2</sub>-Resin depicts an optional solid phase and  
 its coupling arm to the synthetic peptide.

- Synthesis of the present peptides need not be  
 30 limited to the Boc and FMOC synthetic techniques depicted  
 above. For example benzyloxycarbonyl-leu-norleucinal

-15-

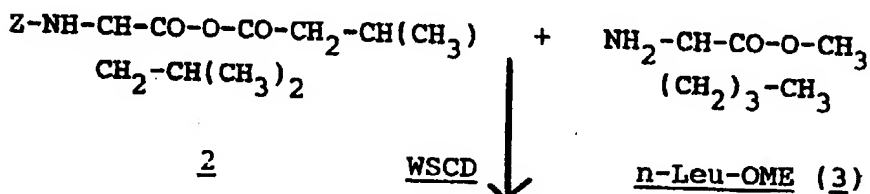
- 1 (calpeptin) may be synthesized in solution, using  
 benzyloxycarbonyl-leucine (Z-Leu-OH, 1) as starting  
 material. Z-Leu-OH is first reacted with  
 isobutylchloroformate to make a mixed anhydride as  
 5 depicted below.



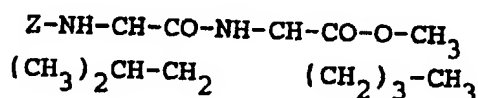
15

Mixed Anhydride (2)

- The mixed anhydride (2) is then coupled with the methyl  
 ester of norleucine (n-Leu-OMe, 3) using a coupling  
 20 reagent, for example, N-ethyl-N',N'-dimethylaminopropyl  
 carbodiimide (WSCD).



25



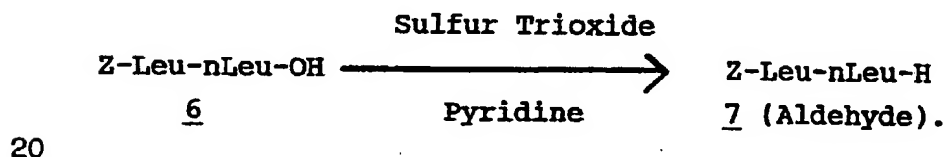
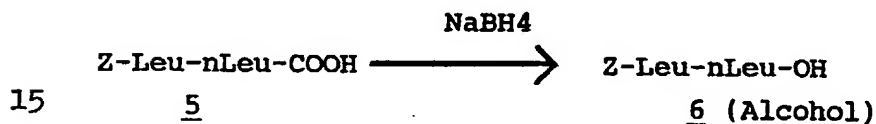
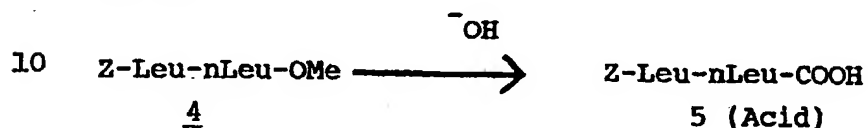
30

Z-Leu-nLeu-OMe (4)

35

-16-

1 The ester, Z-Leu-nLeu-OMe(4), can be saponified by  
reaction with a strong base to form the acid (5).  
Reduction with, for example sodium borohydride, yields  
the corresponding alcohol (6) and subsequent partial  
5 oxidation with sulfur trioxide pyridine complex,  
triethylamine and dimethylsulfoxide, generates the  
corresponding aldehyde (7). These reactions are depicted  
below:



The above synthetic reactions illustrate general and specific techniques for synthesizing peptides, and for generating the aldehyde or alcohol derivatives from the C-terminal carboxylic acids.

25 Substituting the C-terminal carboxylic acid for diazomethane can be accomplished by first converting the carboxylate group into a mixed anhydride using, for example, isobutyl chloroformate in tetrahydrofuran and N-ethyl morpholine. This reaction mixture can then be

30 added to ethereal diazomethane and allowed to react overnight. The diazomethane derivatized peptide may then

35

- 1 be extracted with water and isolated. Chloromethane  
derivatized peptides may be made from the diazomethane  
derivative by treatment of the later with HCl in ethanol  
at 0°C (Green et al., 1981, J. Biol. Chem. 256:  
5 1923-1928; Sasaki, et al., 1986, J. Biochem. 99:  
173-179).

In some cases the alpha amino group of the  
subject peptide may be amidated by addition of a R'-CO-  
group, e.g., acyl group, or an epoxysuccinyl group. This  
10 can be done before, during or after peptide synthesis.  
Amidation may be accomplished by any of the art  
recognized procedures. For example, amide formation  
frequently involves acylation of the amine with acid  
chlorides, anhydrides, esters or carboxylic acids. The  
15 reaction with carboxylic acids may be of limited utility  
unless the acid is first activated so the -OH group  
becomes a good leaving group. Carbodiimides can be used  
to activate the carboxylic acid. Common acylating  
procedures for amide formation employ acid chlorides or  
20 anhydrides in pyridine.

Addition of a cholesteryl or an aryl group to  
the N-terminal amino acid is most easily done before the  
peptide is synthesized. This also prevents unwanted side  
reactions with non-N-terminal amino acid side groups.  
25 Blocking groups may be used on the N-terminal amino acid  
side group and carboxylate, if needed. By using the  
blocking groups described for use in peptide synthesis,  
the cholesteryl- or aryl-derivatized N-terminal amino  
acid may then be used directly in peptide synthesis.  
30 Cholesterol or any aryl group may be derivatized at a  
reactive position to provide a good leaving group. For

1 example, the C-3 alcohol of cholesterol may be  
derivatized with p-toluenesulfonyl chloride  
(p-CH<sub>3</sub>-C<sub>6</sub>H<sub>4</sub>-SO<sub>2</sub>Cl), thionyl chloride (SOCl<sub>2</sub>) or be  
5 replaced with a halide or other group to provide a  
leaving group. Aryl groups with appropriate leaving  
groups are frequently commercially available. The  
appropriately blocked amino acid is then reacted with the  
derivatized cholesterol or aryl group under conditions  
10 which allow the leaving group to be replaced by the  
N-terminal amine.

After synthesis, peptides may be purified and  
salts may be removed by gel electrophoresis, flash  
chromatography with a silica gel column, or by other size  
selective chromatography procedures, including gel  
15 chromatography or high-pressure liquid chromatography.

The present invention is directed to peptides  
of the instant general formulae which have utility to  
inhibit undesired cellular growth. Hence these peptides  
are useful, for example, in the therapy or prevention of  
20 cancer, for prevention or treatment of arterial occlusion  
(arteriosclerosis) and for inhibiting smooth muscle or  
endothelial cell proliferation.

Accordingly, the present invention provides a  
method of inhibiting animal cell proliferation by  
25 administering an effective amount of at least one of the  
hydrophobic peptides defined in accordance with this  
invention. An effective amount is that amount which is  
sufficient to inhibit cell growth or proliferation,  
particularly in cells which are in an inappropriate or  
30 excessive growth phase, or an amount to retard or prevent  
the course of a disease state.

1           In particular, the present method is useful for  
inhibiting cell proliferation whether it occurs in vivo  
or in vitro. When this method is used for in vivo cell  
inhibition, the method can include administering the  
5   subject peptides to an animal such as mammals, e.g.  
humans, monkeys, rabbits, mice, cows, or a veterinary  
animal or farm animal such as cats, dogs, chickens,  
turkeys, horses and the like. By in vitro cell  
proliferation is meant the inhibition of cultured cells,  
10   e.g. tissue culture cells or primary cell cultures  
obtained from a patient. Hence the method of inhibiting  
cultured cells can include administering, treating or  
co-culturing such cells in the presence of the subject  
peptide. For example, the peptides may be co-cultured  
15   with cells continuously or for varying periods of time.

          Similarly, the subject peptides can be  
administered singly or in combination as dictated by the  
exigency of the condition. One skilled in the art can  
readily select and combine the subject peptides for  
20   simultaneous administration if necessary or as required  
by the circumstances of treatment.

          The present method is particularly useful for  
inhibiting smooth muscle or endothelial cell  
proliferation. Consequently, this method provides a  
25   means of treating and preventing diseases associated with  
inappropriate, inopportune, or excessive cell  
proliferation of smooth muscle or endothelial tissues.  
In particular, such diseases include arteriosclerosis,  
restenosis and prostatic hypertrophy. The treatment and  
30   prevention of restenosis by the present method is

1 especially useful after percutaneous transluminal  
coronary angioplasty.

Moreover, the present method is particularly  
useful for inhibiting the proliferation of cancerous  
5 cells in an animal or in cell culture. Hence, the method  
provides a means of treating and preventing cancer and  
cancer-like diseases, e.g. benign tumors, arising from  
inappropriate, inopportune or excessive cell  
proliferation.

10 The administration of the subject peptides can  
be accomplished by any convenient route known to those  
skilled in the art by providing the subject peptides in  
an effective amount sufficient to inhibit cell  
proliferation as required by the exigency of the therapy.  
15 Routes of administration include oral, enteric,  
parenteral, intravenous, intramuscular, intrapericardial,  
intranasal and topical. The amount of peptide delivered  
varies by route and can be determined by one skilled in  
the art in accordance with the guidelines provided  
20 herein, especially as relates to pharmaceutical  
compositions.

The present peptides inhibit cellular  
proliferation both in vitro and in vivo. Cultured cells  
can be used to test for the effects of the present  
25 peptides on the rate of cell division. The rate of cell  
division can be assessed by exposing cells to the subject  
peptides, then counting cell number as a function of time  
and comparing to controls. Addition of the subject  
peptides to the cell culture medium causes a significant  
30 decrease in cell growth. Cells grown without the subject  
peptides increase in number by almost 10-fold in four

-21-

1 days. Cells grown in the presence of the subject peptides show little or no increase in number over a similar time period.

5 The rate of DNA synthesis can be determined by observing the incorporation of labeled nucleotide into cellular DNA. A nucleotide may be radioactively labeled. Alternatively, a labeled nucleotide may be a nucleotide analog that is readily incorporated into DNA and is detected by a second detector molecule. One example of a  
10 nucleotide analog is bromodeoxyuridine (BUdr) which may be detected by, for example, fluorescently labeled antibodies directed against bromodeoxyuridine. Total DNA content may be assessed, for example, by using propidium iodide.

15 To assess which stage of the cell cycle is affected by the present antiproliferative agents, cells may be synchronized or reversibly arrested in the  $G_0$ , or non-growing stage, of the cell cycle. Synchronization can be achieved by any of a number of reagents known to  
20 one skilled in the art, or simply by replacement of the culture medium with serum-free medium for 2 or more days. To initiate synchronized cell growth the cells may be rinsed with rich medium containing no cell cycle arresting reagent and then allowed to grow in normal,  
25 serum containing medium. To test the affect that the present peptides have on the growth of synchronized cells, the peptides may be added to the growth medium at various times after removal of the cell cycle arresting reagent or after addition of serum to the medium, i.e.,  
30 after releasing the cell cycle arrest.

35

-22-

1           To assess the cell cycle stage of the cells,  
flow cytometry can be used to sort cells on the basis of  
DNA content. Cells which have incorporated more than a  
5       distinguished, counted and sorted from cells with only a  
normal 2n DNA content. DNA content is a reflection of a  
cell's stage in the cell cycle. A 2n DNA content is the  
normal content for a cell which is not synthesizing DNA.  
Hence a cell with a 2n DNA content may be a non-growing  
10       cell, or a cell which has just divided but not yet begun  
DNA synthesis for another round of cell division. A 4n  
DNA content indicates that the cell has finished  
duplicating its normal DNA content but has not yet  
physically divided into two daughter cells. A DNA  
15       content between 2n and 4n indicates a cell is in the  
process of DNA synthesis.

Cells exposed to the subject peptides for short  
periods of time do not progress through the DNA synthesis  
(S) phase of the cell cycle and hence have a 2n DNA  
20       content long after cell cycle synchronization. However,  
when exposed to the present peptides for up to 2 weeks  
some cells may undergo DNA synthesis (i.e., have a DNA  
content greater than 2n) but do not undergo cell  
division, indicating that the present peptides can cause  
25       a further block in the cycle at the G<sub>2</sub> or M phase.

In a further analysis of the biochemistry of  
cells exposed to the peptides of this invention, the  
present invention demonstrates that transcription of two  
known cellular proto-oncogenes, c-fos and c-myc, are  
30       depressed relative to that observed in cells not exposed  
to the present peptides. The c-fos and c-myc gene

35

1 products are believed to act as transcription factors  
having a role in transformation of a cell to a malignant  
phenotype. Hence, the present peptides are useful not  
only for depressing cell growth but also for depressing  
5 the production of factors that can lead to malignant  
cancer.

The active ingredients of the present  
pharmaceutical compositions include the present peptides  
which exhibit antiproliferative activity when  
10 administered in therapeutic amounts from about 0.1 mg to  
about 2000 mg per kg of body weight per day and  
preferably in amounts of from about 1.0 to 100 mg per kg  
of body weight. Localized administration of a 1.0 to  
1000 micromolar solutions of the subject peptides is  
15 preferred. One skilled in the art can adjust the dosage  
regimen to provide the optimum therapeutic response. For  
example, one daily dose may be administered or several  
divided doses may be given and the doses may be  
proportionally reduced or increased as indicated by the  
20 exigencies of the therapeutic situation. The active  
compound may be administered in a convenient manner such  
as by an intravenous, intraparacardial, oral,  
intramuscular, intradermal, or subcutaneous route. The  
active compounds may also be administered parenterally or  
25 intraperitoneally. Intravenous or intrapericardial  
administration is preferred.

Depending on the route of administration, the  
active ingredients of the subject pharmaceutical  
composition may be coated in a material to protect the  
30 ingredients from the action of enzymes, acids or other  
natural products. Dispersions can be prepared in

1 glycerol, liquid polyethylene glycols, oils and in  
mixtures thereof. Under ordinary conditions of storage  
and use, these preparations may contain a preservative to  
prevent the growth of microorganisms.

5 The pharmaceutical preparations suitable for  
injection include sterile liposomal suspensions, aqueous  
solutions or dispersions, as well as sterile powders for  
extemporaneous preparation of injectable solutions or  
dispersions. In all cases the preparation must be  
10 sterile and must be fluid to the extent that it is easily  
syringable. A preparation must be stable under the  
conditions of manufacture and storage and must be  
preserved against the contaminating action of  
microorganisms such as bacteria and fungi. The carrier  
15 can be a solvent or dispersion medium containing, for  
example, water, ethanol, polyol (for example, glycerol,  
propylene glycol, liquid polyethylene glycol, and the  
like), suitable mixtures thereof, and vegetable oils.  
The proper fluidity can be maintained, for example, by  
20 the use of a coating such as lecithin, by the maintenance  
of the required particle size in the case of dispersion  
and by the use of surfactants. The prevention of  
microbial action can be brought about by various  
antibacterial and antifungal agents, for example,  
25 parabens, chlorobutanol, phenol, sorbic acid, thimerosal,  
and the like. In many cases, it is preferable to include  
isotonic agents, for example, sugars or sodium chloride.

Preferred carriers are those which protect the  
active compound against rapid elimination from the body,  
30 such as controlled release formulations, including  
implants and microencapsulated delivery systems.

1 Biodegradable, biocompatible polymers can be used, such  
as polyanhydrides, polyglycolic acid, collagen and  
polylactic acid.

5 Liposomal carriers are also contemplated as  
preferred carriers by the present invention. In addition  
to the subject peptides, liposome carriers may  
incorporate within them agents which help target the  
subject peptides to the appropriate cell type, e.g.  
10 antibodies directed against membrane proteins found only  
on a specific cell type. Liposomal formulations may be  
prepared by dissolving appropriate lipids in an inorganic  
solvent which is subsequently evaporated to generate a  
thin film of dried lipid on the surface of a container.  
15 Appropriate lipids may include stearoyl phosphatidyl  
ethanolamine, stearoyl phosphatidyl choline, arachadoyl  
phosphatidyl choline and cholesterol. An aqueous  
solution of the active compound, with the desired  
additional carriers or agents as described above, is then  
20 introduced into the container. The container is then  
swirled to free the lipids from the sides of the  
container and to disperse lipid aggregates, thereby  
forming the liposomal suspension. Methods for  
preparation of liposomal formations will be apparent to  
those skilled in the art.

25 Sterile injectable solutions may also be  
prepared by incorporating the active compounds in the  
required amount and in the appropriate solvent with  
various of the other ingredients enumerated above,  
followed by filter sterilization. Generally, dispersions  
30 are prepared by incorporating the various sterilized  
active ingredients into a sterile vehicle which contains

1 the basic dispersion medium and the required other  
ingredients (enumerated above). In the case of sterile  
powders for the preparation of sterile injectable  
solutions, the preferred method of preparation is vacuum  
5 drying. This freeze-drying technique yields a powder of  
the active ingredient with any additional desired  
ingredients from the previously sterile-filtered  
solution.

When the present peptides are suitably  
10 protected they may be orally administered, for example,  
with an inert diluent or with an assimilable edible  
carrier, or they may be enclosed in hard or soft gelatin  
capsule. They may also be compressed into tablets, or  
15 incorporated directly into a food which is part of the  
diet. For oral therapeutic administration, the active  
peptide may be incorporated with excipients and used in  
the form of ingestible tablets, buccal tablets, troches,  
capsules, elixirs, suspensions, syrups, wafers, and the  
like. Such compositions and preparations should contain  
20 at least 1% of active compound. The percentage of the  
compositions and preparations may, of course, be varied  
and may conveniently be between about 5% to about 80% of  
the weight of the unit. The amount of active compound in  
such therapeutically useful compositions is such that a  
25 suitable dosage is obtained. Preferred compositions or  
preparations according to the present invention are  
prepared so that an oral unit dosage form contains  
between about 1  $\mu$ g and 2000 mg of active compound.

The tablets, troches, pills, capsules and the  
30 like may also contain the following: a binder such as  
gum tragacanth, acacia, corn starch or gelatin;

1    excipients such as dicalcium phosphate; a disintegrating  
agent such as corn starch, potato starch, alginic acid  
and the like; a lubricant such as magnesium stearate; and  
a sweetening agent such as sucrose, lactose or saccharin  
5    may be added or a flavoring agent such as peppermint, oil  
or wintergreen, or cherry flavoring. When the dosage  
form is a capsule, it may contain, in addition to  
materials of the above type, a liquid carrier. Various  
other materials may be present as coatings or to  
10   otherwise modify the physical form of the unit dosage.  
For instance, tablets, pills, or capsules may be coated  
with shellac. A syrup or elixir may contain the active  
compound, sucrose as a sweetening agent, methyl and  
propylparabens as preservatives, a dye and flavoring such  
15   as cherry or orange flavor. Of course, any material used  
in preparing any dosage unit form should be  
pharmaceutically pure and substantially non-toxic in the  
amounts employed. In addition, the active compound may  
be incorporated into sustained-release preparations and  
20   formulations.

It is especially advantageous to formulate  
parenteral compositions in dosage unit form for ease of  
administration and uniformity of dosage. Unit dosage  
form as used herein refers to physically discrete units  
25   suitable as unitary dosages for the mammalian subjects to  
be treated; each unit containing a predetermined quantity  
of active material calculated to produce the desired  
therapeutic effect in association with the required  
pharmaceutical carrier. The specification for the novel  
30   dosage unit forms of the invention are dictated by and  
directly dependent on (a) the unique characteristics of

-28-

1 the active material and the particular therapeutic effect  
to be achieved, and (b) the limitations inherent in the  
art of compounding such an active material for the  
prevention of disease in living subjects.

5 The principal active peptide ingredient, is  
compounded for convenient and effective administration in  
pharmaceutically effective amounts with a suitable  
pharmaceutically acceptable carrier in dosage unit form  
as hereinbefore disclosed. A unit dosage form can, for  
10 example, contain the principal active compound in amounts  
ranging from 1 ug to about 2000 mg. Expressed in  
proportions, the active compound is generally present in  
from about 1 ug to about 2000 per ml of carrier. In the  
case of compositions containing supplementary active  
15 ingredients, the dosages are determined by reference to  
the usual dose and manner of administration of the said  
ingredients.

As used herein, "pharmaceutically acceptable  
carrier" includes any and all solvents, dispersion media,  
20 coatings, antibacterial and antifungal agents, isotonic  
and absorption delaying agents, and the like. The use of  
such media and agents for pharmaceutical active  
substances is well known in the art. Except insofar as  
any conventional media or agent is incompatible with the  
25 active ingredient, its use in the therapeutic  
compositions is contemplated. Supplementary active  
ingredients can also be incorporated into the  
compositions.

The following examples further illustrate the  
30 invention.

35

1

EXAMPLE 1Materials and MethodsCell Culture

5 Bovine aortic smooth muscle cells were obtained  
by outgrowth from medial explants of thoracic aortae of  
cows within 4 hours of slaughter. Initial outgrowth as  
well as standard maintenance growth was in DMEM with 10%  
FBS added. Media were renewed every 2-3 days. All  
10 growth was in a humidified incubator equilibrated with a  
5% CO<sub>2</sub> atmosphere. Cultures were passaged immediately  
prior to full confluence by brief exposure to HBSS  
(Hank's Balanced Salt Solution) containing trypsin (0.5  
mg/ml) and EDTA (0.5 mM); all experiments were performed  
15 using cells of passage 7 or less. As a test of  
viability, cells were counted and assessed for trypan  
blue exclusion with a hemocytometer at each passage and  
at selected times during time course experiments,  
routinely showing >95% of the population to exclude  
trypan blue. For most subcultures and all experiments  
20 cells were plated at a density of 10,000 cells/cm<sup>2</sup>,  
regardless of container.

These cells exhibited typical morphologic  
characteristics of vascular smooth muscle in vitro  
including a pattern of variably multilayered growth, and  
25 demonstrated specific immunoperoxidase staining by a  
monoclonal antibody selective for muscle  $\alpha$ -actin (HHF-35)  
which did not react with endothelial cells, and is known  
not to stain fibroblasts (Tsukada, et al., 1987, Am. J.  
Pathol. 127:51-60).

30

35

1 [<sup>3</sup>H] Thymidine Incorporation

[<sup>3</sup>H] thymidine incorporation was used to assess cell growth. After obtaining cells as described above they were seeded in 24-well microtiter plates and allowed to attach overnight. The cultures were washed with PBS and placed in serum-free medium, consisting of DMEM with 1  $\mu$ M insulin and 5  $\mu$ g/ml transferrin added, for a total of 48-52 hours (more than one doubling time for actively cycling cells). During the last 6 hours of this incubation, agents were added at variable concentrations in a carrier solution consisting of 1:1 EtOH: H<sub>2</sub>O to a final EtOH concentration of 0.5 mg per 100 ml. Serum-free medium was then removed and replaced by DMEM with 10% FBS using concentrations of agent and carrier solutions identical to those in the serum-free medium. [<sup>3</sup>H] thymidine was added 18 hours after serum repletion to a concentration of 2  $\mu$ Ci/ml, and was incubated for 6 additional hours. At the end of the incubation, cells were released from the wells and incorporated precursor removed by washing with distilled water. Cell residues were collected on glass mesh filter by a automated cell harvester. Radioactivity was measured by liquid scintillation spectroscopy.

Flow Cytometric Assays

Cells were plated, allowed to attach, and placed in serum free media for 48-52 hours as described above. Compounds to be tested were added at the designated concentrations in the serum-free media followed by serum-containing media, also as described above. At the specified times after serum addition, these cells were harvested by trypsin/EDTA, washed with

- 1 HBSS/5% BSA, pelleted, and resuspended in PBS containing  
0.6% NP-40 and 0.1 mg/ml propidium iodide, to which was  
added RNase to a final concentration of 2mg/ml. Flow  
cytometric analyses were done using a Becton-Dickinson  
5 FACScan wherein the exciting wavelength was at 488 nm and  
the detecting wavelength at 585 nm. Events were gated on  
a fluorescence-area vs.-width map to eliminate potential  
clumped nuclei. Such events represented less than 3% of  
the total in general.

10 Calpeptin Synthesis

- Calpeptin was synthesized using an adaptation  
of the protocol described by Tsujinaka et al. (1988,  
Biochem. Biophys. Res. Comm. 153:1201-1208). Briefly,  
Z-leu-OH was reacted with isobutyl chloroformate to form  
15 a mixed anhydride intermediate; this was then coupled to  
nLeu-OMe-HCl, followed by saponification with 1 N NaOH to  
yield Z-Leu-nLeu-OH. This was reduced to the alcohol  
with sodium borohydride, and then partially oxidized to  
the corresponding aldehyde with sulfur trioxide/pyridine.  
20 The overall yield starting from the original components  
was 26% after purification by flash chromatography with a  
silica gel column using initially 20% then 30% ethyl  
acetate in hexanes mixture. Following recrystallization  
from hot hexanes, the melting point was 90-93°C. Purity  
25 was confirmed by TLC in 1:1 ethyl acetate:hexanes.

30

35

1

EXAMPLE 2The Present Peptides are Antiproliferative  
Agents for Cells in Culture

Cells were cultured as described in Example 1.

5 To assess cell growth, either tritiated thymidine or a deoxynucleotide analog, bromodeoxyuridine (BUdr) was added to the medium; the incorporation of tritiated thymidine or of fluorescently labeled antibodies directed against bromodeoxyuridine, respectively, gave a measure of cell proliferation. Addition of propidium iodide to the medium allowed determination of the total amount of DNA. Cells were sorted by flow cytometry to distinguish populations of cells with different DNA (i.e., in this example, BUdr) contents.

15

Addition to the medium of benzyloxycarboxyl-Leu-norleucinal at 100  $\mu$ M or acetyl-Leu-Leu-norleucinal at 50  $\mu$ M caused a significant decrease in cell growth. As depicted in Fig. 2, the number of cells normally increased almost 10-fold in 4 days after serum addition but addition of calpeptin (benzyloxycarbonyl-Leu-norleucinal) resulted in almost no increase in cell number.

20

Removal of serum from the medium was used to synchronize the cell cycle of cultured cells (Fig. 3), thereby allowing an assessment of which phase of the cell cycle was affected by the compounds tested. As depicted in Fig. 1, cells normally require about 24 hr to progress through the cell cycle. After serum depletion almost all cells are arrested in the G<sub>0</sub>/G<sub>1</sub> phase with a 2n DNA content (a normal, non-dividing amount of DNA). As depicted in Fig. 3, untreated cells exhibit little

25

30

35

1 increase in DNA content at 12 hr after addition of serum.  
However, by 18 hr a significant number of untreated cells  
have more than a 2n DNA content, indicating that DNA  
synthesis is progressing. By 21 hr, approximate equal  
5 numbers of cells have a 2n or 4n DNA content, while the  
remaining cells have a DNA content between 2n and 4n. By  
24 hr almost all untreated cells have a 4n or 2n DNA  
content. Cells with a 4n DNA content are likely undergo  
cell division shortly, while those with a 2n DNA content  
10 may have just undergone cell division.

Fig. 4 depicts the effect of calpeptin on the  
DNA content of cells. In the absence of calpeptin and 24  
hr after serum addition to serum-depleted cells, more  
cells have a 4n DNA than a 2n DNA content, indicating  
15 that significant DNA synthesis is occurring in these  
cells. However, cells exposed to calpeptin do not  
progress through the DNA synthesis phase of the cell  
cycle, as demonstrated by the 2n DNA content of almost  
all calpeptin exposed cells in Fig. 4. By 2 weeks after  
20 serum addition most non-exposed cells have a 2n DNA  
content indicating that they have stopped growing. Many  
calpeptin-exposed cells, on the other hand, have a 4n DNA  
content at 2 weeks after serum addition, indicating that  
some cell cycle progression has occurred, and that an  
25 additional block has occurred in these cells at the G<sub>2</sub> or  
M phase of the cell cycle. No increase in aneuploidy of  
DNA content (over 4n) was observed relative to controls.  
Cell counting demonstrated that no cell growth had  
occurred in 2 weeks in cells exposed to calpeptin or  
30 acetyl-Leu-Leu-norleucinal and that there were less than  
20% nonviable cells at any time. The EC<sub>50</sub> of calpeptin

- 1 and acetyl-Leu-Leu-norleucinal is 56 and 14  $\mu$ M,  
respectively.

Fig. 5 depicts the effects of different  
concentrations of calpeptin, acetyl-Leu-Leu-norleucinal  
5 (Inhibitor I of Fig. 5) and acetyl-Leu-Leu-methioninal  
(Inhibitor II of Fig. 5) on DNA synthesis in  
proliferating aortic smooth muscle cells as measured by  
tritiated thymidine incorporation by smooth muscle cells.  
Cells exposed to either calpeptin or acetyl-Leu-Leu-  
10 norleucinal incorporate significantly less tritiated  
thymidine than do control cells or cells exposed to a  
synthetic peptide which is not an anti-proliferative  
agent (Inhibitor II).

15

20

25

30

35

1

EXAMPLE 3The Present Peptides Inhibit Transcription  
of Proto-Oncogenes

5 By Northern analysis of total cellular mRNA  
produced after addition of serum to serum depleted cells,  
acetyl-leucine-leucine-norleucinal caused a 4-fold  
decrease, relative to controls, in the expression of  
c-fos and c-myc, known cellular proto-oncogenes. A  
similar decrease in actin mRNA was observed upon addition  
10 of acetyl-leucine-leucine-norleucinal. In all cases,  
mRNA was obtained from the same number of cells. The  
production of mRNA from adenine phosphoribosyl-  
transferase (APRT), a housekeeping gene required for  
general metabolic function in all cells, was used as an  
15 internal standard against which different mRNA  
preparations could be compared. No decrease in APRT mRNA  
was seen in treated cells, relative to untreated cells.  
Hence, these peptides not only inhibit cell proliferation  
but also depress the synthesis of cellular oncogenes.  
20

25

30

35

1

EXAMPLE 4Prevention of Restenosis After Angioplasty  
by the Present Peptides

5 Acetyl-Leu-Leu-norleucinal has been shown to  
reduce proliferation of cells in culture (Example 2). To  
demonstrate that acetyl-Leu-Leu-norleucinal and related 0  
peptides can have a similar effect in vivo, and be useful  
in the prevention of arteriosclerosis and restenosis  
after angioplasty, arteriosclerotic rabbits were treated  
10 with acetyl-Leu-Leu-  
norleucinal immediately after angioplasty. Prevention of  
restenosis after angioplasty was used as a test system  
for prevention of arteriosclerosis because the underlying  
mechanisms of restenosis are like those occurring during  
15 arteriosclerotic plaque formation.

A 50  $\mu$ M solution of acetyl-Leu-Leu-norleucinal  
was slowly injected into 7 arteriosclerotic rabbit  
femoral arteries immediately following angioplasty. A  
porous Wolinsky catheter was used for this injection to  
20 slowly disperse the peptide into the artery for a period  
of 45 sec. As a control, five contralateral arteries  
were injected with the carrier solution containing no  
acetyl-Leu-Leu-norleucinal (Control 1). As a further  
control, 12 femoral arteries were treated only with  
25 angioplasty (Control 2). All animals were sacrificed  
after angiography, which was done 2 weeks after  
angioplasty. Arteries were sectioned into 3mm segments,  
stained with hematoxylin/eosin and elastin/trichrome and  
morphometry was performed. Results were evaluated by  
30 analysis of variance.

35

1                   Table 1 summarizes the results of the study.  
Angiography showed that the luminal diameter of animals  
treated with acetyl-Leu-Leu-norleucinal was larger than  
5   in controls, i.e. there was a smaller decrease after  
angioplasty in treated than in non-treated animals.  
Similarly, histological measurements indicated that  
treated animals had a larger lumen area than control  
animals. Neointimal area was the same for all groups  
10   ( $0.76 \pm 0.42 \text{ mm}^2$  for control 1 animals and  $0.63 \pm 0.31$   
 $\text{mm}^2$  for control 2 animals;  $p = 0.817$ ). However, the  
neointima/media ratio was smaller in  
acetyl-Leu-Leu-norleucinal treated animals due to an  
increased medial thickness. These data demonstrate that  
injection of these peptides reduces post-angioplasty  
15   restenosis while preserving the medial layer.

20

25

30

35

1

TABLE 1

Effect of Acetyl-Leu-Leu-norleucinal  
(Inhibitor 1) on Restenosis after Angioplasty

5	Treatment	Decrease	Luminal	Medial
	after	in arterial	area (by	Thickness
	angioplasty	lumina (by	histology)	
		angioplasty		
10	None	$0.83 \pm 0.36 \text{ mm}$	$0.54 \pm 0.26 \text{ mm}^2$	$0.38 \pm 0.05$
	Injection	$0.88 \pm 0.33 \text{ mm}$	$0.55 \pm 0.19 \text{ mm}^2$	$0.45 \pm 0.14$
	with			
	Carrier			
15	Solution			
	Injection	$0.26 \pm 0.27 \text{ mm}$	$0.84 \pm 0.40 \text{ mm}^2$	$0.76 \pm 0.38$
	with			
	Inhibitor 1			
20	Statistical	$p = 0.004$	$p = 0.113$	$p = 0.02$
	significance			
	(check)			

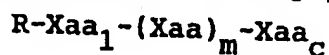
25

30

35

1 WHAT IS CLAIMED IS:

1. A method of inhibiting animal cell proliferation which comprises administering an effective amount of a hydrophobic peptide sufficient to inhibit cell growth, wherein said peptide has the formula:



wherein:

m is 0-5;

R is hydrogen, epoxysuccinyl, cholesteryl, aryl, aralkyl or acyl;

Xaa<sub>1</sub> and (Xaa)<sub>m</sub> are independently an amino acid selected from the group consisting of Ala, Arg, Ile, Leu, Lys, Met, nLeu, Phe, Pro, Thr, Tyr, Trp and Val;

Xaa<sub>c</sub> is an amino acid selected from the group consisting of Ala, Arg, Cys, Ile, Leu, Lys, nLeu, Phe, Pro, Thr, Tyr, Trp and Val, or the corresponding alcohol, aldehyde, epoxysuccinate, acid halide, carbonyl halomethane or diazomethane derivative of the carboxy terminal group of said amino acid.

2. The method of Claim 1 wherein m is 1.

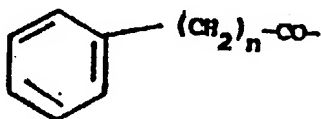
3. The method of Claim 1 wherein m is 0.

4. The method of Claim 2 or 3 wherein Xaa<sub>1</sub> is Leu, Val or nLeu.

5. The method of Claim 2 wherein Xaa<sub>m</sub> is Leu or Val.

6. The method of Claim 2 or 3 wherein Xaa<sub>c</sub> is Lys, Leu, nLeu, Phe or Tyr.

7. The method of Claim 2 or 3 wherein R is acetyl, benzyloxycarbonyl, cholesteryl, epoxysuccinyl, phenyl or



1 wherein n is 0 to 6.

8. The method of Claim 2 wherein the hydrophobic peptide is acetyl-Leu-Leu-norleucinal.

5 9. The method of Claim 3 wherein the hydrophobic peptide is benzyloxycarbonyl-Leu-norleucinal.

10. The method according to any one of Claims 1, 2, 3, 8 or 9 wherein said cell proliferation is inhibited in smooth muscle cells.

10 11. The method according to any one of Claims 1, 2, 3, 8 or 9 wherein said cell proliferation is inhibited for the treatment of prostatic hypertrophy.

12. The method according to Claims 1, 2, 3, 8 or 9 wherein said cell proliferation is inhibited for the treatment of arteriosclerosis.

15 13. The method according to any one of Claims 1, 2, 3, 8 or 9 wherein said cell proliferation is inhibited for the treatment of restenosis.

20 14. The method of Claim 13 wherein said restenosis follows percutaneous transluminal coronary angioplasty.

15. The method according to any one of Claims 1, 2, 3, 8 or 9 wherein said cell proliferation is inhibited for the treatment of cancerous cell proliferation.

25 16. A pharmaceutical composition for inhibiting cell proliferation comprising a therapeutic amount of at least one hydrophobic peptide and a pharmaceutically acceptable carrier, wherein said peptide has the formula:

30 
$$R-Xaa_1-(Xaa)_m-Xaa_c$$
  
and further wherein:

35

1

m is 0-5;

R is hydrogen, epoxysuccinyl, cholesteryl, aryl, aralkyl or acyl;

5

Xaa<sub>1</sub> and (Xaa)<sub>m</sub> are independently an amino acid selected from the group consisting of Ala, Arg, Ile, Leu, Lys, Met, nLeu, Phe, Pro, Thr, Tyr, Trp and Val;

10

Xaa<sub>c</sub> is an amino acid selected from the group consisting of Ala, Arg, Cys, Ile, Leu, Lys, nLeu, Phe, Pro, Thr, Tyr, Trp and Val, or the corresponding alcohol, aldehyde, epoxysuccinate, acid halide, carbonyl halomethane or diazomethane derivative of the carboxy terminal group of said amino acid.

15

17. The composition of Claim 16 wherein said peptide is acetyl-Leu-Leu-norleucinal or benzyloxycarbonyl-Leu-norleucinal.

18. The composition of Claim 16 wherein said peptide is present in an amount to provide from about 0.1 mg to about 2000 mg per kilogram of body weight per day.

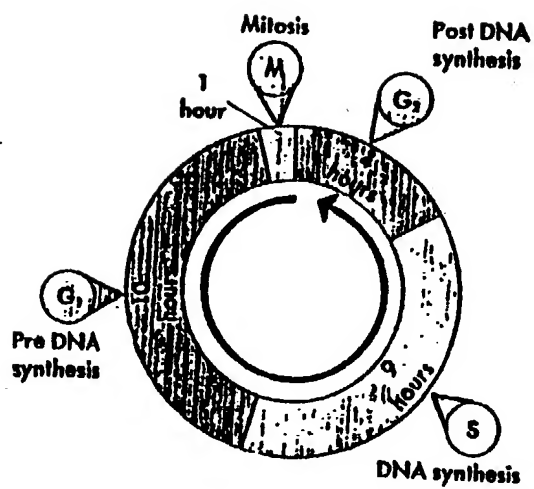
20

25

30

35

Figure 1



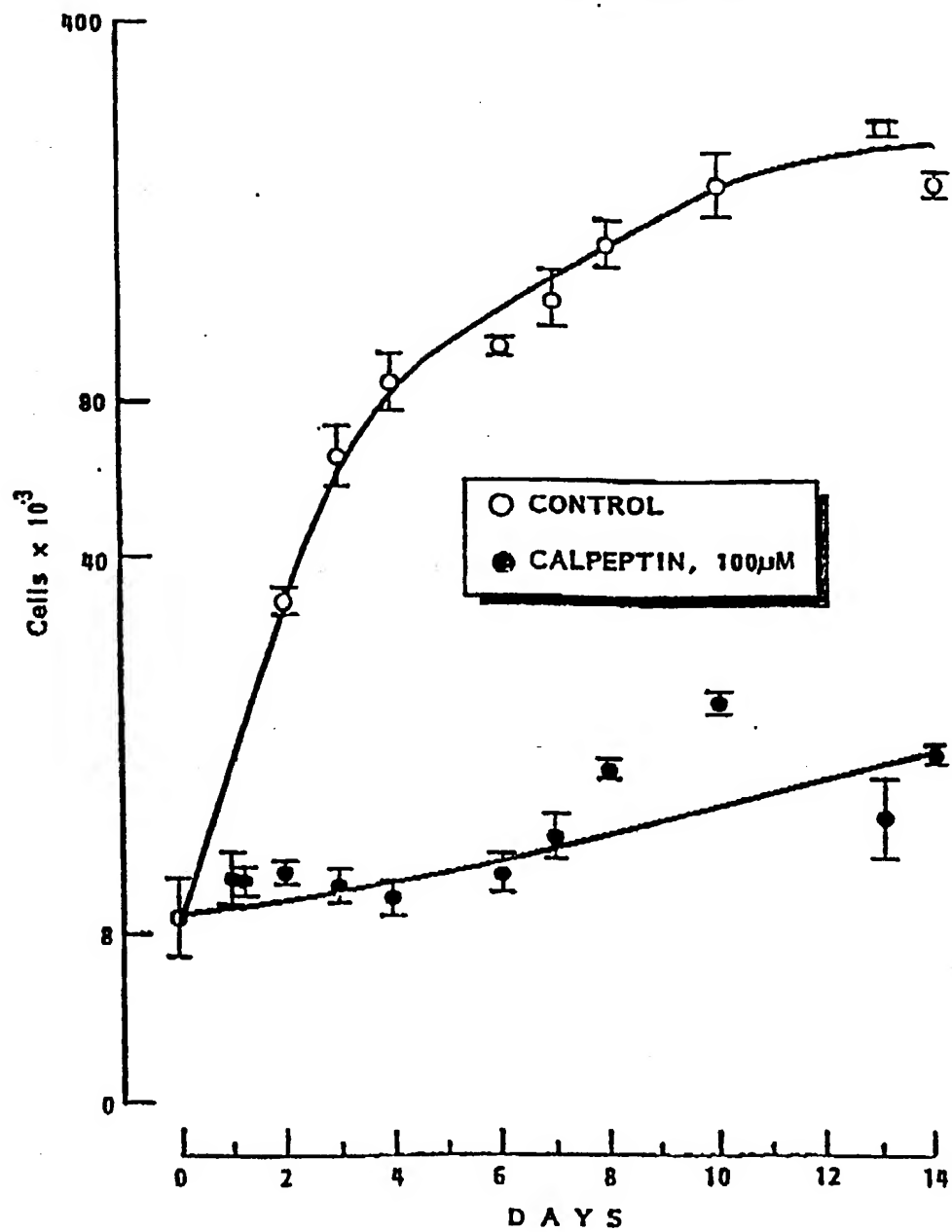
Effects of Calpeptin on Proliferation of  
Vascular Smooth Muscle Cells

Figure 3

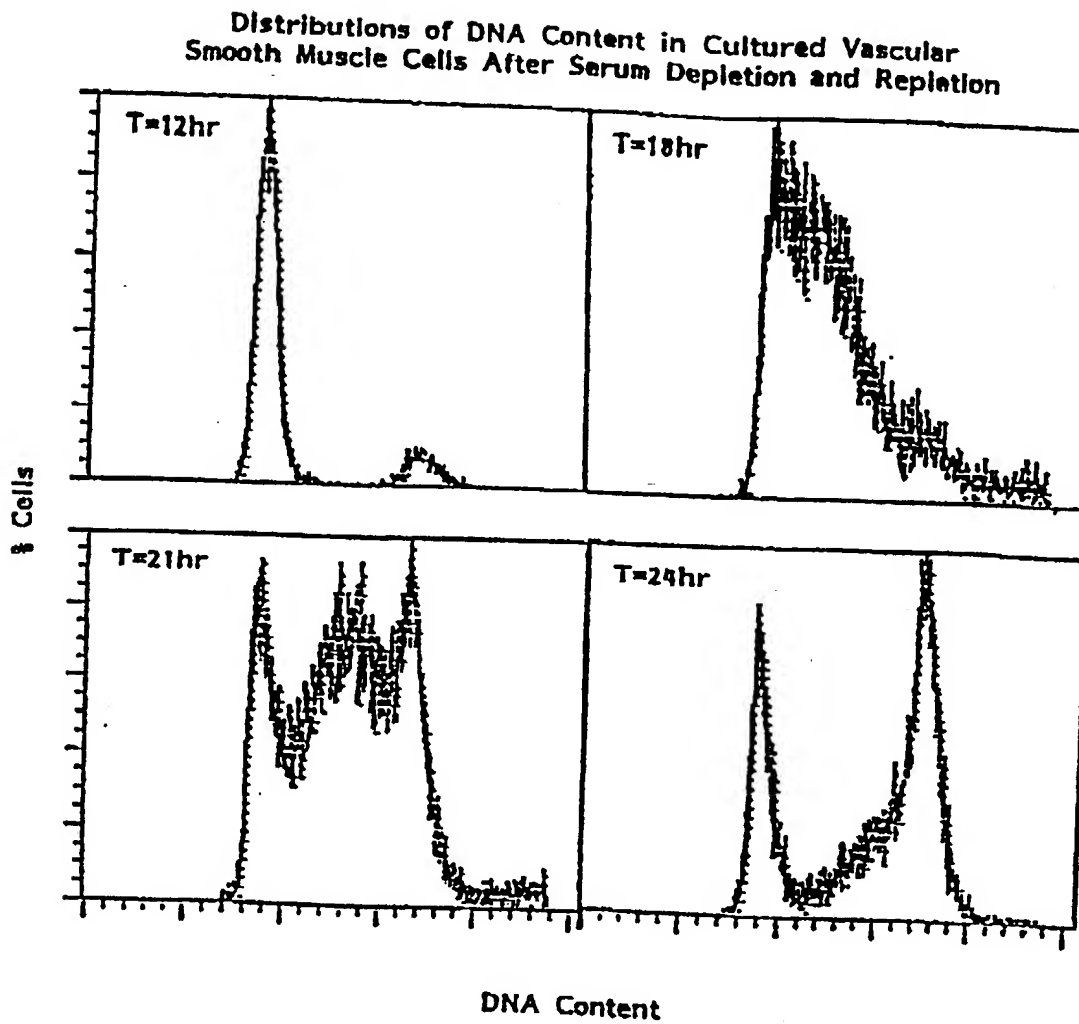


Figure 4

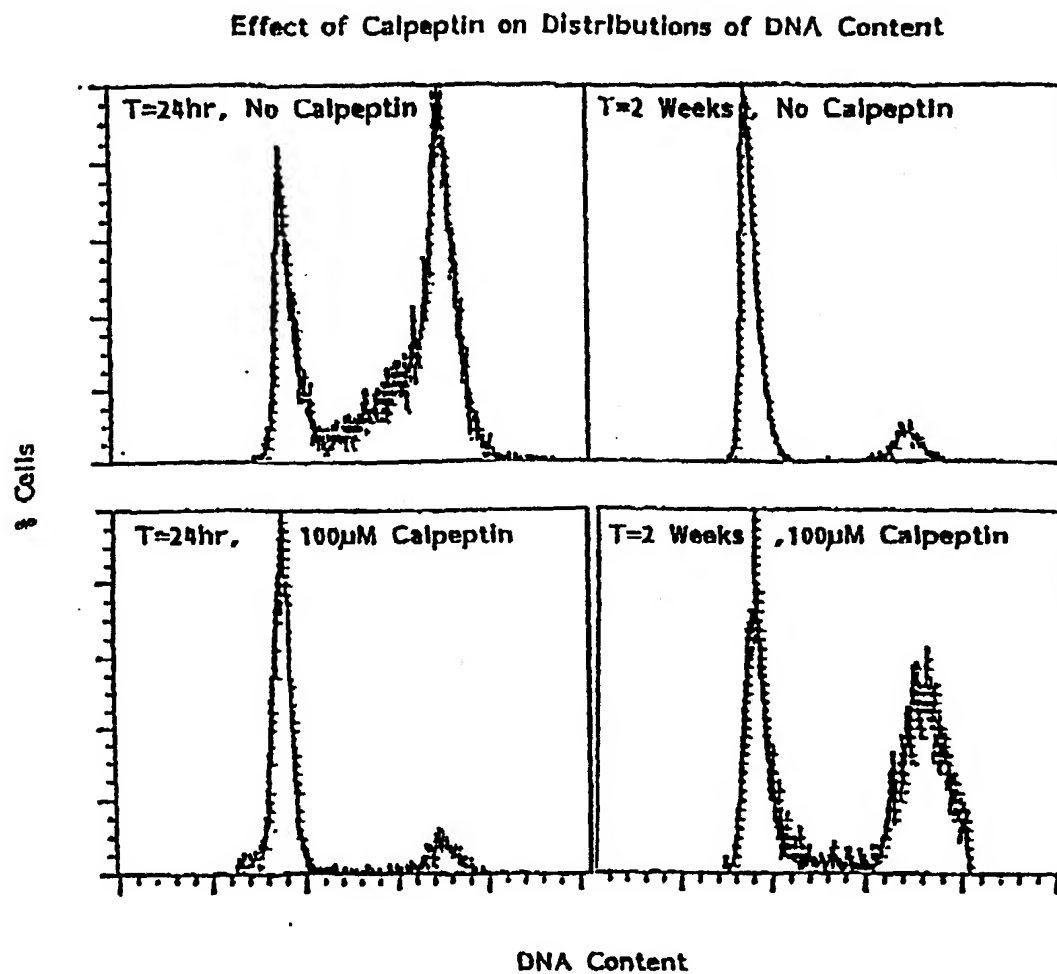
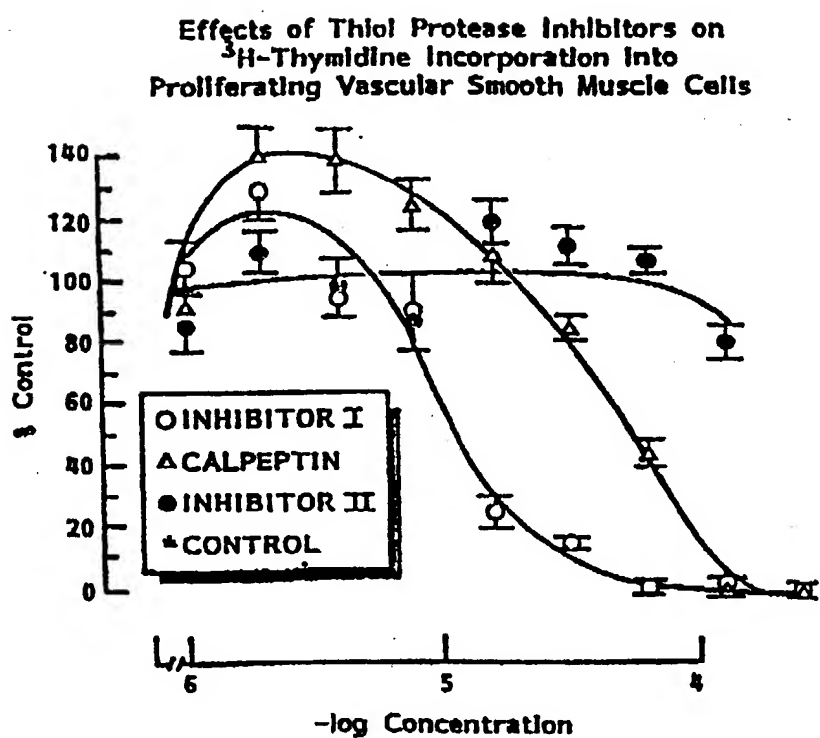


Figure 5




# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00905

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>3</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC (5): A61K 37/02; C07K 5/00		
US CL : 530/329,330,331; 514/17,18,19		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>4</sup>		
Classification System	Classification Symbols	
U.S.	530/329,330,331; 514/17,18,19	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>5</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup>		
Category*	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
X/Y	US, A. 4,752,602 (Lipsky et al.) 21 June 1988, see entire document.	1,3-7,16,18/9
X/Y	EP, A. 0,383,190 (Guindon et al.) 22 August 1990, see entire document.	1,10,16,18/ 2,8,11,12,13,1 4,15,16-18
Y	Biochemical And Biophysical Research Communications, Volume 153, No.3, issued 30 June 1988, Tsujinaka et al. "Synthesis Of A New Cell Penetrating Calpain Inhibitor (Calpeptin)", pages 1201-1208, see tables I and II on pages 1204-1205.	16-18
Y	JP A, 61-103897 (Murachi et al.) 22 May 1986 See entire document.	16-18
Y	Blood, Volume 76, No. 12, issued 15 December 1990, Fox et al., "The Role of Calpain in Stimulus-Response Coupling: Evidence That Calpain Mediates Agonist-Induced Expression of Procoagulant Activity In Platelets", pages 2510-2519, see entire article.	16-18
<p>* Special categories of cited documents:<sup>15</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search <sup>2</sup>	Date of Mailing of the International Search Report <sup>2</sup>	
05 MAY 1992	21 MAY 1992	
International Searching Authority <sup>1</sup>	Signature of Authorized Officer <sup>20</sup>	
ISA/US	Deborah F. Fouse for Bennett Celsa	

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00906

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>3</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC (5): A61K 37/02; C07K 5/00 US CL : 530/329,330,331; 514/17,18,19		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>4</sup>		
Classification System	Classification Symbols	
U.S.	530/329,330,331; 514/17,18,19	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>5</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup>		
Category <sup>6</sup>	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
X/Y	US, A. 4,752,602 (Lipsky et al.) 21 June 1988, see entire document.	1,3-7,16,18/9
X/Y	EP, A. 0,383,190 (Guindon et al.) 22 August 1990, see entire document.	1,10,16,18 / 2,8,11,12,13,1 4,15,16-18
Y	Biochemical And Biophysical Research Communications, Volume 153, No.3, issued 30 June 1988, Tsujinaka et al. "Synthesis Of A New Cell Penetrating Calpain Inhibitor (Calpeptin)", pages 1201-1208, see tables I and II on pages 1204-1205.	16-18
Y	JP A. 61-103897 (Murachi et al.) 22 May 1986 See entire document.	16-18
Y	Blood, Volume 76, No. 12, issued 15 December 1990, Fox et al., "The Role of Calpain in Stimulus-Response Coupling: Evidence That Calpain Mediates Agonist-Induced Expression of Procoagulant Activity In Platelets", pages 2510-2519, see entire article.	16-18
<p>* Special categories of cited documents:<sup>15</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search <sup>2</sup>	Date of Mailing of the International Search Report <sup>2</sup>	
05 MAY 1992	21 MAY 1992	
International Searching Authority <sup>1</sup>	Signature of Authorized Officer <sup>20</sup>	
ISA/US	 Bennett Celsa	